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Chaperonin overproduction and metabolic erosion caused by mutation accumulation in *Escherichia coli*

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We dedicate this manuscript to the memory of Mario A. Fares.

Running title: GroEL overproduction and metabolic erosion in *E. coli*

Abstract

Bacterial cells adapting to a constant environment tend to accumulate mutations in portions of their genome that are not maintained by selection. This process has been observed in bacteria evolving under strong genetic drift, and especially in bacterial endosymbionts of insects. Here, we study this process in hypermutable *Escherichia coli* populations evolved through 250 single-cell bottlenecks on solid rich medium in a mutation accumulation experiment that emulates the evolution of bacterial endosymbionts. Using phenotype microarrays monitoring metabolic activity in 95 environments distinguished by their carbon sources, we observe how mutation accumulation has decreased the ability of cells to metabolize most carbon sources. We study if the chaperonin GroEL, which is naturally overproduced in bacterial endosymbionts, can ameliorate the process of metabolic erosion, because of its known ability to buffer destabilizing mutations in metabolic enzymes. Our results

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Key words: Metabolism; experimental evolution; molecular chaperones; bacterial endosymbionts.

Introduction

Organisms adapting to a constant environment for many generations tend to lose fitness in other environments, because they do not benefit from maintaining fitness in environments they no longer encounter (Mills *et al.*, 1967; Futuyma, 1988; Bennett & Lenski, 1993; Fong *et al.*, 1995). This evolutionary process is known as ecological specialization, and it can be either driven by natural selection or by genetic drift (Cooper & Lenski, 2000; Cooper, 2014; Leiby & Marx, 2014). Specialization can be driven by natural selection if an adaptive phenotype shows antagonistic pleiotropy—a phenotype that is selected for being optimal in a given environment is deleterious in other environments due to physiological trade-offs. Alternatively, mutations that are neutral in a particular selective environment can be deleterious in other environments, and such mutations can accumulate in a genome, especially in "unused" genomic regions, by random genetic drift. These two causes of ecological specialization are not mutually exclusive.

Specialization through neutral mutations is especially important in bacterial endosymbionts of some insects such as aphids (Moya *et al.*, 2008; Toft & Andersson, 2010; McCutcheon & Moran, 2012). These bacteria live inside host cells that provide a highly constant environment, and have been evolving clonally for millions of generations in this environment. Endosymbionts produce nutrients missing from the host diet, while benefiting from the nutrient-rich and stable intracellular environment of the host. They are maternally inherited by the host, and experience severe bottlenecks during their vertical transmission from generation to generation, which result in small effective population sizes and strong genetic drift. Consequently, natural selection is considerably less efficient in these bacteria than in free-living bacteria. Additionally, a lack of functional DNA repair enzymes (which results in high mutation rates) further accelerate the accumulation of mutations in their genomes, and a lack of recombination prevents the effective purging of deleterious mutations (Moran, 1996).

As a consequence of their high mutational load, such endosymbionts have evolved a mechanism to buffer deleterious mutations. Specifically, they overproduce proteins that help fold other proteins,

molecular chaperones such as GroEL and DnaK, which can reduce the fitness cost of destabilizing mutations in proteins (Baumann *et al.*, 1996; Fares *et al.*, 2002; Wilcox *et al.*, 2003; McCutcheon *et al.*, 2009; Stoll *et al.*, 2009; McCutcheon & Moran, 2012; Bennett & Moran, 2013; Fan *et al.*, 2013; Oakeson *et al.*, 2014). In the rich chemical environment that their host cells provide, selection on many metabolic genes is relaxed in these endosymbionts (Moya *et al.*, 2008; Moran *et al.*, 2009; Toft & Andersson, 2010; Latorre & Manzano-Marín, 2017). Therefore, these genes tend to accumulate loss-of-function mutations that cause metabolic erosion, a process by which an organism loses metabolic abilities, such as the ability to metabolize nutrients.

In the Long-Term Evolution Experiment (LTEE), 12 populations of *Escherichia coli*—the closest free-living relative to the well-studied endosymbiont of aphids *Buchnera aphidicola* (Moran & Wernegreen, 2000)—have evolved for more than 60,000 generations (and counting) in a minimal medium containing glucose as the only source of carbon and energy with an effective population size of $\sim 3.3 \times 10^7$ (Lenski *et al.*, 1991), which is larger than in bacterial endosymbionts. Adaptation to this single environment has resulted in decreased performance in other environments (Cooper & Lenski, 2000; Leiby & Marx, 2014). Some populations have evolved hypermutable (mutator) phenotypes (Sniegowski *et al.*, 1997). In these populations the higher mutation rate caused substantial metabolic erosion, measured as the number of different chemical environments where fitness has declined. This suggests that rather than physiological trade-offs underlying antagonistic pleiotropy, the neutral process of mutation accumulation suffices to cause metabolic specialization in the LTEE (Leiby & Marx, 2014). Additionally, the fitness reduction of mutator populations is ameliorated by growth at a lower temperature. Most amino acid substitutions are slightly deleterious because of their destabilizing effects, and destabilizing mutations tend to increase a protein's heat sensitivity (DePristo *et al.*, 2005). Therefore, temperature-dependent fitness reduction suggests that in these populations metabolic erosion is caused by destabilizing mutations affecting metabolic enzymes (Leiby & Marx, 2014). If this is the case, the overproduction of chaperones that can buffer mutations affecting protein stability could have a similar effect to low temperature in ameliorating metabolic erosion caused by mutation accumulation.

GroEL is a member of the family of chaperones known as chaperonins, which are large double-ring complexes that enclose target proteins for folding within a cylindrical folding chamber (Hartl *et*

al., 2011). GroEL can buffer destabilizing mutations in its target proteins (Fares *et al.*, 2002; Maisnier-Patin *et al.*, 2005; Tokuriki & Tawfik, 2009; Bogumil & Dagan, 2010; Williams & Fares, 2010; Bershtein *et al.*, 2013; Wyganowski *et al.*, 2013; Pechmann & Frydman, 2014; Sabater-Muñoz *et al.*, 2015). Many of these proteins are metabolic enzymes (Takemoto *et al.*, 2011). Enzymes evolved under GroEL overproduction accumulate twice as many mutations, and these mutations have higher destabilizing effects than in the absence of GroEL overproduction (Tokuriki & Tawfik, 2009). These observations make it plausible that chaperones such as GroEL could slow down the process of metabolic erosion in endosymbionts.

One of the first demonstrations that GroEL can buffer deleterious mutations came from *E. coli* populations with high mutational loads (Fares *et al.*, 2002). These populations had evolved for more than 3,000 generations via single-cell bottlenecks on solid glucose-limiting minimal medium. Half of the populations evolved at a high mutation rate. The evolution of these populations was dominated by genetic drift, due to the extreme bottlenecks they experienced during their evolution, which are similar to bottlenecks experienced by endosymbionts during their vertical transmission between hosts. At the end of the experiment, the evolved populations had considerably reduced their fitness (growth rate) in the experimental environment. Moreover, overproducing GroEL in the evolved populations restored fitness to almost ancestral levels. This restoration was observed after supplementing the minimal growth medium with amino acids, otherwise, the energetic cost of overproducing GroEL was so great that no fitness recovery was observed. Similar results were later obtained in mutator populations of *Salmonella typhimurium* (Maisnier-Patin *et al.*, 2005). Both studies focused on a single environment. In this study, we investigate whether GroEL overproduction can ameliorate the metabolic erosion experienced by bacteria evolved under conditions that favor the accumulation of mutations in a large number of environments. To address this question, we study mutator *E. coli* populations evolved for thousands of generations under conditions that emulate those of bacterial endosymbionts evolution and we assay their ability to metabolize different carbon substrates using phenotype microarrays.

Results

Our experiment begins with three independent clonal populations from a mutation accumulation experiment that had been initiated from the same mutator clone (*E. coli* K12 MG1655 Δ*mutS*). The populations had been evolved by daily passaging through single-cell bottlenecks on solid rich growth

(LB) medium at 37 °C (Alvarez-Ponce *et al.*, 2016; Sabater-Muñoz *et al.*, 2017) for 250 days or approximately 5,500 generations (~22 generations between bottlenecks). In such an evolution experiment, the efficiency of natural selection is severely reduced because of the extreme bottlenecks to which the evolving lineages are exposed. In consequence, non-lethal mutations can accumulate freely under the influence of genetic drift (Barrick & Lenski, 2013).

We transformed the ancestor and a clone from each of the three evolved populations with the plasmid pGro7 (Nishihara *et al.*, 1998). This plasmid contains the operon *groE*, which encodes GroEL, and its co-chaperone GroES, under the regulation of a single promoter inducible by L-arabinose. We also transformed the ancestor and each of the three evolved clones with a control plasmid pGro7-DEL(*groE*) (pGro7c) that lacks the operon *groE* but is otherwise identical to pGro7 (Materials and Methods). In the presence of the expression inducer L-arabinose, only cells harboring the plasmid pGro7 overproduce GroEL. In total, we thus generated eight strains from the three evolved clones (E1, E2, and E3) and their ancestor (A). Four of these strains contain the plasmid pGro7 (A/pGro7, E1/pGro7, E2/pGro7, and E3/pGro7), and the other four contain the control plasmid pGro7c (A/pGro7c, E1/pGro7c, E2/pGro7c, and E3/pGro7c). L-arabinose cannot be used as a carbon source by any of these strains.

To study metabolic erosion in the evolved populations we used Biolog phenotype microarrays, which allow the high-throughput measurement of growth and cellular respiration in multiple environments contained in different wells of a 96-well microtiter plate (Bochner *et al.*, 2001; Bochner, 2009). These arrays measure metabolic activity using tetrazolium, a redox dye that absorbs the electrons from the electron transport chain, and changes color when being reduced by respiring cells. Biolog assays are widely used, and have been useful to map the phenotypes of various genotypes (e.g., gene knock-out mutants) (Bochner *et al.*, 2001; Zhou *et al.*, 2003; Pommerenke *et al.*, 2010), to study metabolic innovation (Toll-Riera *et al.*, 2016), to study macroevolutionary patterns of phenotypic evolution in bacteria (Plata *et al.*, 2015), or to characterize the phenotypes of experimentally evolved lineages (Le Gac *et al.*, 2012; Hug & Gaut, 2015). Specifically, we used PM1 plates, which measure cellular respiration and growth in 95 chemical environments distinguished by their carbon source, to determine the metabolic phenotypes for each of the eight strains used in this study (Materials and Methods, Table S1).

We define metabolic erosion as the fraction of carbon sources that an evolved population metabolizes more slowly than the ancestor strain from which the mutation accumulation experiment started. To measure metabolic erosion, we only consider carbon sources that the ancestor was able to metabolize above a minimum threshold that is defined by measurement noise (Materials and Methods). We consider that a metabolic phenotype has not declined if it is above this noise threshold, and if it is not significantly lower than the ancestral phenotype (*t*-test, false discovery rate < 0.05, Materials and Methods). To evaluate the extent of this erosion in the evolved populations, we first focused on the strains that harbor the control plasmid pGro7c, that is, they do not overproduce GroEL. After having experienced 250 single-cell bottlenecks, that is, ~5,500 generations of mutation accumulation, the evolved populations (E1/pGro7c, E2/pGro7c, and E3/pGro7c) fare considerably worse than their ancestor (A/pGro7c) in a majority of the carbon sources tested (between 100% to 90.9%; Table 1).

Table 1. Metabolic erosion in the presence and absence of GroEL overproduction for each of three populations evolved in a mutation accumulation experiment for more than 5,000 generations.

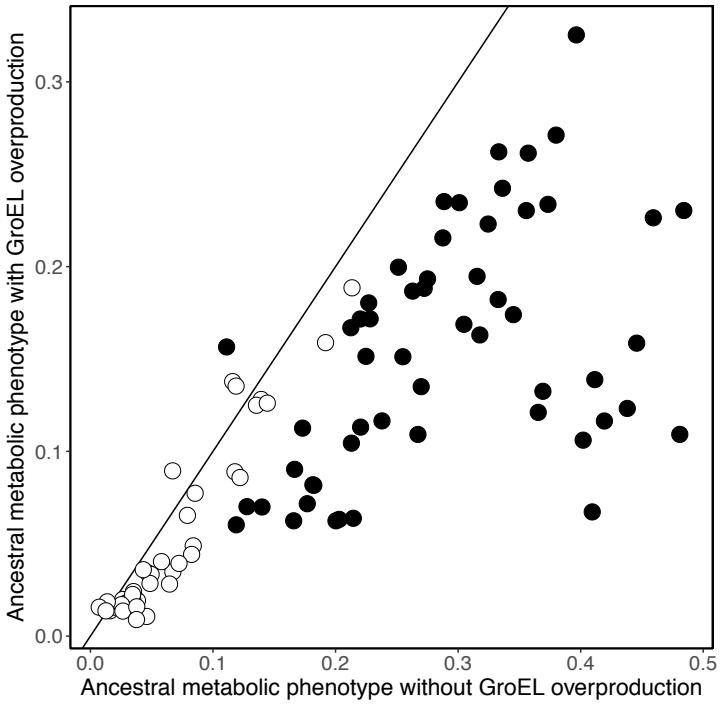
Evolved population	**Plasmid**[a]	**Metabolic erosion**[b]
E1	pGro7c	98.7% (76/77)
E1	pGro7	88.6% (62/70)
E2	pGro7c	90.9% (70/77)
E2	pGro7	75.7% (53/70)
E3	pGro7c	100% (77/77)
E3	pGro7	95.7% (67/70)

[a]Cells harboring the plasmid pGro7 overproduce GroEL in the presence of the expression inducer L-arabinose, while cells harboring the control plasmid pGro7c do not.
[b]The fraction of those carbon sources on which evolution caused significant metabolic erosion, based on carbon sources which the ancestor metabolizes above the noise threshold of 0.039 out of 95 tested carbon sources (Materials and Methods).

The evolved lineages E1 and E2 had been sequenced in a previous study (Alvarez-Ponce *et al.*, 2016). Analyzing the sequence data, we find that metabolic erosion and the number of accumulated nonsynonymous mutations are not associated in a straightforward pattern. For example, population E1 accumulated 303 mutations and metabolizes 98.7% of carbon sources more poorly than the ancestor,

while population E2 accumulated more (731) mutations and shows metabolic erosion on fewer (90.9%) carbon sources. Of the 304 and 731 nonsynonymous mutations accumulated in lineages E1 and E2, 139 and 277 mutations (45.9% and 37.9%, respectively) affected metabolic enzymes (Keseler *et al.*, 2013). Lineages E1 and E2 had 182 and 224 metabolic pathways (43% and 53%, respectively) affected by nonsynonymous mutations (Keseler *et al.*, 2013). The observation that E2 cells express GroEL and DnaK more highly than E1 cells may help explain the poor correlation between metabolic erosion and the number of accumulated mutations. Similarly to GroEL, DnaK is a chaperone that can help buffer the effects of mutation, and that is highly expressed in bacterial endosymbionts (Aguilar-Rodríguez *et al.*, 2016; Kadibalban *et al.*, 2016).

To find out whether chaperones can indeed mitigate metabolic erosion, we turned to the mutation accumulation populations that overproduce GroEL. To analyze data from these populations, however, we needed to take into account that chaperone overproduction carries energetic costs (Fares *et al.*, 2002; Sabater-Muñoz *et al.*, 2015). The ATP consumed in the reaction cycle of the chaperonin contributes less to this cost than the synthesis of large amounts of GroEL and GroES proteins (Fares *et al.*, 2002; Bogumil & Dagan, 2012). The cost is evident by comparing the ancestral strains A/pGro7 with A/pGro7c, where GroEL overproduction causes a decline in metabolic rates in most environments (Fig. 1). To take this cost into account, we compared the metabolic phenotypes of evolved strains overexpressing GroEL (E1/pGro7, E2/pGro7, and E3/pGro7) with the corresponding ancestor (A/pGro7). We find that populations overexpressing GroEL show less metabolic erosion than populations with no GroEL overproduction (Table 1). However, this reduction in metabolic erosion is small (it occurs in only 9.9% of environments on average; e.g., L-Arabinose, D-Galactose, D-Gluconic Acid and L-Glutamic Acid for both E1 and E2) because the number of environments where there is no decline of the metabolic phenotype with GroEL overproduction is small (average = 9.3). We observe that populations with a higher level of metabolic erosion show lower amelioration of this erosion after GroEL overproduction (Table 1). However, this correlation is not significant (Pearson's correlation coefficient = -0.910, $P = 0.273$), probably because it is based on little data (n=3).



186

187 **Figure 1. GroEL overproduction incurs metabolic costs.** Metabolic phenotypes of the ancestor on
188 95 different carbon sources. Each circle represents the rate at which the ancestor can metabolize one of
189 the carbon sources averaged over four technical replicates of the ancestor without GroEL
190 overproduction (horizontal axis) and with GroEL overproduction (vertical axes). The diagonal line
191 indicates equal metabolic rates with and without GroEL overproduction. Circles below the diagonal
192 line represent carbon sources where GroEL overproduction causes a reduction in metabolic rate. Filled
193 circles indicate environments where the difference in metabolic rate with and without chaperonin
194 overproduction is statistically significant, i.e., greater than experimental measurement noise (Materials
195 and Methods).

196 **Discussion**

197 Bacteria evolving for many generations in the same chemical environment experience a process of
198 metabolic specialization by which they lose or reduce their ability to metabolize many different
199 metabolic substrates. Arguably, bacterial endosymbionts provide the best well-known examples of this
200 process (Latorre & Manzano-Marín, 2017). In some cases, the decline of metabolic phenotypes in
201 these bacterial species can cause substantial fitness reductions in the host (Latorre & Manzano-Marín,
202 2017). Mutation accumulation experiments mimic some of the conditions present during the evolution
203 of intracellular bacteria, and bacteria evolved in this type of experiment acquire some characteristics
204 common to endosymbionts (Sabater-Muñoz *et al.*, 2017), such as high mutational loads (Lee *et al.*,
205 2012; Sabater-Muñoz *et al.*, 2015; Aguilar-Rodríguez *et al.*, 2016), genome reduction (Nilsson *et al.*,

206 2005), or higher expression of molecular chaperones such as GroEL and DnaK (Maisnier-Patin *et al.*,
207 2005; Sabater-Muñoz *et al.*, 2017). In this study, we analyzed mutator populations subject to daily
208 single-cell bottlenecks for more than 5,000 generations in a nutrient-rich environment. At the end of
209 this mutation accumulation experiment, these bacterial populations showed similar patterns of genome
210 reduction, mutational biases, and gene expression (Sabater-Muñoz *et al.*, 2017).

211 Previous studies of bacteria evolved in mutation accumulation experiments have shown that
212 they can experience great fitness reductions, which can be partially compensated by the
213 overproduction of chaperones (Fares *et al.*, 2002; Maisnier-Patin *et al.*, 2005; Sabater-Muñoz *et al.*,
214 2015; Aguilar-Rodríguez *et al.*, 2016). However, these studies did not explore fitness reduction in
215 environments different from the environment in which evolution took place. Here, using phenotype
216 microarrays, we assayed evolved populations in 95 different environments distinguished by their
217 carbon sources. We find that bacterial populations evolved under strong genetic drift can lose or
218 reduce their ability to metabolize many distinct carbon sources. Because these populations have
219 evolved under conditions that considerably reduce the power of natural selection to drive the fixation
220 of beneficial mutations, the most likely cause for metabolic erosion is the accumulation of mutations
221 driven by genetic drift, rather than the fixation of advantageous mutations that show antagonistic
222 pleiotropy (Leiby & Marx, 2014).

223 However, we note that there is no correlation between the number of accumulated
224 nonsynonymous mutations and metabolic erosion in two of the evolved populations for which we have
225 whole-genome sequences from a previous study (Alvarez-Ponce *et al.*, 2016). There is also no
226 correlation between the number of nonsynonymous mutations in metabolic enzymes, and metabolic
227 erosion. There are at least two possible explanations for why the relationship between mutational load
228 and erosion may not be straightforward. First, neutral or slightly deleterious mutations in metabolic
229 enzymes can be highly pleiotropic because of the organization of metabolism as a reaction network. A
230 loss-of-function mutation in a highly connected enzyme can simultaneously and negatively affect
231 several distinct metabolic phenotypes. The second non-exclusive possibility is that the relationship
232 between mutational load and erosion may be influenced by chaperone expression. We find that the
233 population with a higher chaperone expression shows less metabolic erosion (Sabater-Muñoz *et al.*,
234 2017). The effect of chaperone expression on metabolic erosion may be mediated through the

buffering of destabilizing mutations in metabolic enzymes (Tokuriki & Tawfik, 2009). Such mutations may have also caused the erosion observed in populations from the LTEE (Leiby & Marx, 2014).

GroEL and the Hsp70 chaperone DnaK are the most abundant proteins in the cytosol of endosymbiotic bacteria (Baumann *et al.*, 1996; Wilcox *et al.*, 2003; McCutcheon *et al.*, 2009; Stoll *et al.*, 2009; McCutcheon & Moran, 2012; Bennett & Moran, 2013; Fan *et al.*, 2013; Oakeson *et al.*, 2014). For instance, GroEL expression is 7.5 times higher in *B. aphidicola* than in its close free-living relative *E. coli* (Baumann *et al.*, 1996). These observations suggest that GroEL and DnaK help these cells cope with their high mutational loads by buffering the negative effects of destabilizing mutations in proteins, and this hypothesis has experimental support (Fares *et al.*, 2002; Maisnier-Patin *et al.*, 2005; Sabater-Muñoz *et al.*, 2015; Aguilar-Rodríguez *et al.*, 2016). Here, we find some further evidence that chaperones could ameliorate some of the negative effects of metabolic erosion. In particular, we observe that evolved populations overproducing the chaperonin GroEL show less metabolic erosion than in the absence of chaperonin overproduction. However, we only observe this mitigating effect of GroEL overproduction in a few environments. This is not remarkable if we consider that GroEL is not an all-powerful molecular machine, capable of buffering any possible deleterious mutation. The buffering ability of GroEL is most certainly restricted to certain proteins and types of mutations (Wang *et al.*, 2002; Kerner *et al.*, 2005; Fujiwara *et al.*, 2010; Tartaglia *et al.*, 2010; Williams & Fares, 2010). However, it would be worth exploring if GroEL overproduction could show greater amelioration of metabolic erosion in populations evolved for fewer generations, and therefore with smaller mutational loads. Another interesting avenue for further work would be to study metabolic erosion with different expression levels of GroEL to better map the trade-off between expression cost and buffering benefit (Sabater-Muñoz *et al.*, 2015).

In summary, we show that bacterial populations evolving in conditions emulating the evolution of bacterial endosymbionts experience a severe reduction in their ability to metabolize many distinct carbon substrates. We find that the relationship between this metabolic erosion and mutational load is not straightforward, and could be influenced by both pleiotropy and chaperone expression. Finally, we show that GroEL overproduction can mitigate metabolic erosion in some environments. In so doing, we provide evidence that molecular chaperones can reduce in more than one environment the negative impact of genome decay on cell metabolism.

Materials and Methods

Strains and plasmids

Strains E1, E2, and E3 derive from E. coli K12 substr. MG1655 Δ*mutS*. They evolved in parallel in a long-term mutation accumulation experiment on solid LB medium at 37 °C (Alvarez-Ponce *et al.*, 2016; Sabater-Muñoz *et al.*, 2017), where each population was passaged for 250 days through a single-cell (clonal) bottleneck after 24 hours of growth. Passaged colonies were chosen randomly.

We derived the control plasmid pGro7c from the plasmid pGro7 (Takara, Cat. #3340) via PCR amplification using primers TGATAACTCTCCTTTGAGAAAGTCCG and TTGCCCTGCACCTCGCAGAAATAA, and Phusion® High-Fidelity DNA polymerase (NEB), after having obtained permission from Takara to modify pGro7. We digested the PCR products with Dpn1 to remove pGro7. We separated the digested PCR products on a 0.8% agarose gel, excised the band corresponding in size to the desired amplification product, purified the product using the QIAquick Gel extraction kit (50928704, Quiagen), and quantified it using a Nanodrop spectrophotometer. We generated pGro7c by ligating the amplification product using T4 DNA ligase (NEB). We validated the sequence of pGro7c using Sanger sequencing. We transformed the ancestor (A) strain, and the evolved lines (E1, E2, E3) with pGro7 and pGro7c to generate eight different strains: A/pGro7, E1/pGro7, E2/pGro7, E3/pGro7, A/pGro7c, E1/pGro7c, E2/pGro7c, and E3/pGro7c.

Phenotype microarrays

We used Biolog phenotype microarray PM1 (Biolog, Inc., Hayward, California, USA) to assay the carbon utilization phenotypes of each of the eight strains on 95 different carbon sources. Both cell growth and respiration contribute to these metabolic phenotypes, because respiration can be independent of growth (Leiby & Marx, 2014). We performed four replicate Biolog assays for each strain, for a total of $8 \times 4 = 32$ microarrays. To do so, we streaked each strain from glycerol stocks onto LB agar plates supplemented with 25 μg/mL of chloramphenicol (Sigma-Aldrich #C0378). We incubated the plates at 37 °C for 24 hours, and re-streaked the colonies onto fresh plates of the same type, which we incubated at the same temperature for the same period of time. We resuspended the colonies from the latter plates in IF-0 solution (Biolog, Inc., Hayward, California, USA) using sterile cotton swabs, and then centrifuged the suspension at 3,000 g for 3 min. We resuspended the cell pellet in fresh IF-0 to an optical density at 600 nm (OD_{600}) of approximately 0.05 (as measured in a 200 μL

suspension volume). We diluted (1:5) this suspension in IF-0+dye (Biolog, Inc., Hayward, California, USA) supplemented with 25 μg/mL of chloramphenicol, L-methionine (5 μM), cyanocobalamine (0.125 μM), and 0.2% (w/v) L-arabinose (Sigma-Aldrich #3256). We added 100 μL of the resulting solution to each well of a PM1 microarray, which we incubated without shaking at 37 °C for 24 hours. We measured OD_{600} and optical density at 750 nm (OD_{750}) at 0 min and 24 hours using a microplate reader (Tecan Spark 10M). We performed 9 reads per well in a 3-by-3 square grid, and computed the average.

Both the presence of oxidized tetrazolium and that of cells cause absorbance at 600 nm. The absorbance at 600 nm due to the presence of cells can be removed by subtracting the OD_{750} from the OD_{600}, because tetrazolium has almost no absorbance at 750 nm. We therefore used these cell-density corrected values in our study ($OD_{600\text{-}750}$), and computed the metabolic phenotype M_S for substrate S as $OD_{600\text{-}750,24h} - OD_{600\text{-}750,0h}$, where $OD_{600\text{-}750,24h}$ is the corrected optical density after 24 hours, and $OD_{600\text{-}750,0h}$ is the corrected optical density at the start of the experiment. We used a minimum threshold to detect respiration in a given substrate S (Sprouffske *et al.*, 2018). To obtain this threshold we computed the absolute differences between all pairs of 3,072 $OD_{600\text{-}750,0h}$ values (8 strains × 4 replicates × 96 wells). Because at time 0, cells have not started to metabolize yet, these differences must be caused by experimental noise. We only consider differences in M_S as significant if they are greater than 0.039, which is the 98-th percentile of all the differences between wells with no growth.

For each evolved strain we compared the four experimental measurements of M_S with the corresponding measurements for the ancestor using a two-tailed *t*-test. In particular, we compared the three evolved populations with pGro7 (E1/pGro7, E2/pGro7, and E3/pGro7) against A/pGro7, and the three evolved populations with pGro7c (E1/pGro7c, E2/pGro7c, and E3/pGro7c) against A/pGro7c. We adjusted the P values for multiple testing using the false discovery rate method (FDR). We consider that a metabolic phenotype has not declined significantly if $P > 0.05$, and both the evolved and the ancestral average M_S values are greater than 0.039 (the noise threshold).

We quantified metabolic erosion in a given evolved population as

$$\frac{P - C}{P}. \qquad \textbf{(1)}$$

P refers to the number of carbon sources where M_S is greater than the noise threshold in A/pGro7 for evolved strains overexpressing GroEL, and in A/pGro7c for evolved strains that do not overexpress GroEL. *C* refers to the number of carbon sources where the metabolic phenotypes has not declined significantly in the evolved population.

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Supplementary Information

Table S1. Optical density values at 600 nm and 750 nm measured at 0 min and 24 hours per strain and well in a PM1 plate. We report the mean and standard deviation of nine reads per well in a three-by-three square grid, and report the values of four replicates.